

**UNIVERSITÀ DEGLI STUDI DI NAPOLI**

**“FEDERICO II”**

**Scuola di Dottorato in Medicina Molecolare**

**Dottorato di Ricerca in Patologia e Fisiopatologia Molecolare**



**“MicroRNAs signatures of TRAIL resistance in human Non-Small Cell Lung Cancer”**

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**Dipartimento di Biologia e Patologia Cellulare e Molecolare “Luigi  
Califano”**

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## Introduction

### The discovery of microRNAs

In the past years the scientific community was focused mainly on the study of protein coding genes as important players in the pathogenesis of cancer. Little interest has been offered to non-coding RNA genes (ncRNA), heterogeneous class of genes that are transcribed to RNA but not translated to protein. Thirteen years ago a genetic screen in *C. Elegans*, studied to identify genes involved in development timing, resulted in the discovery of the first microRNA (miRNA), *lin-4*, which subsequently was found to interact with the 3' untranslated region of *Lin-14* messenger RNA and to repress its translation. This fascinating form of gene regulation was ignored even during the era of complete genome sequencing, since almost all means of gene identification assume that gene encodes protein.

Seven years later, the second RNA of the miRNAs class, *let-7*, emerged from another *C. Elegans* genetic screen. The discovery of *let-7* was particularly exciting, because its phylogenetic conservation in metazoans implied that miRNAs represented a novel fundamental regulatory class of genes. The discovery of miRNAs coincided in an era in which genetic and mechanistic studies started to unveil the mechanism of small interfering RNA (siRNA), and the connection between the two pathways was soon made.

The understanding of the miRNAs structure fuelled the development of efficient cloning strategies that isolated dozens of miRNAs from diverse eukaryotic species. Meanwhile, many genome projects were completed and the annotated genomes were used to predict miRNAs based on their known features using bioinformatics approaches.

### **Genomics of microRNAs**

Today the miRNA database miRBase ([www.sanger.com](http://www.sanger.com)) contains 4039 miRNA entries from 38 species.

The large number of verified and predicted miRNAs immediately created a new challenge.

Almost 50% of mammalian microRNAs are located in introns of protein coding genes or long ncRNAs transcripts, whereas the remaining part is considerable as independent transcription units with specific promoter core elements and polyadenylation signals [for review see(1-3)]. Among the intragenic miRNAs, 40% are found in introns of protein coding genes, whereas ~10% are located in introns of long ncRNA transcripts. The vast majority of miRNA clusters are single transcription units or overlapped in the same host transcripts, within exons or introns, and in some cases depending on alternative splicing of the host gene, implying that they are transcribed as polycistronic transcripts. Additionally, many miRNAs overlap with two or more transcription units transcribed on opposite DNA strands.

The analysis of miRNAs genomic loci evidences that host genes encoding proteins are involved in a broad spectrum of biological function ranging from embryonic development to the cell cycle and physiology. In addition to

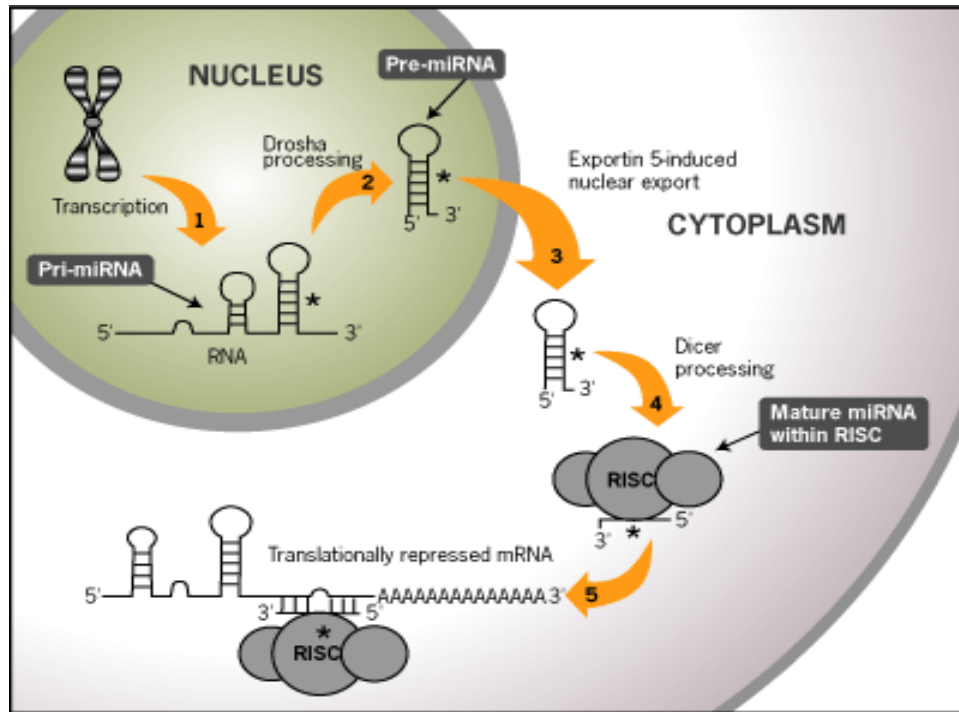
the miRNAs located in protein coding genes, a large group of microRNAs resides in transcripts that lack a significant protein-coding potential, classified as long ncRNAs. These types of ncRNA transcripts are sometimes referred to as mRNA-like ncRNAs (mlncRNA) because they are spliced, polyadenilated and also spatio-temporally expressed. Deleted in Leukemia 2 (DLEU2) and BIC are host-genes mlncRNAs respectively for miR-15/miR-16 cluster and miR-155 (4, 5).

### **microRNAs Transcription and Maturation**

Initially, the researchers believed that microRNAs were transcribed by RNA polymerase III like other small RNAs, as some as tRNAs. However, numerous evidences supported the possibility of a transcription mediated by RNA polymerase II. In 2004, three direct evidences have been reported to evaluate the strict correlation between microRNAs and pol II: (i) the miRNAs transcripts are capped and polyadenilated; (ii) the transcription of miRNAs transcripts is sensitive to alpha-amanitine at the specific concentration for pol II inhibition; (iii) the promoter region, responsible for miRNA transcription, is associated to pol II complex (1). Animal microRNAs are identified as part of 80 nt RNA with stem-loop structure (pre-miRNA) that are included in several hundreds/thousands nucleotide long miRNAs precursors, named primary miRNAs precursor (pri-miRNA) (Figure 1). The production of microRNAs from pri-miRNA to mature miR is a complex and coordinated process where different groups of enzymes and associated proteins, located in the nucleus or cytoplasm, operate the multistep maturation of these tiny RNAs. Principally, the maturation process of microRNAs can be resumed in three important steps: cropping, export and dicing.



In the cropping, the pri-miRNA is converted in pre-miRNA through the cleavage activity of Drosha enzyme, a nuclear Ribonuclease III endonuclease capable to crop the flank regions of pri-miRNA in turn to liberate the 60-70 nt pre-miRNA (6). Different pri-miRNA requisitions are necessary to obtain an efficient precursor maturation by Drosha: first, a large terminal loop (> 10 nucleotides) in the hairpin and a stem region one turn bigger than the pre-miRNA; second, a 5' and 3' single stranded RNA extension at the base of the future microRNAs [for review see (7, 8)].



**Figure 1**

The pri-miRNA is converted in pre-miRNA through the cleavage activity of Drosha enzyme. The resulting product of cropping, the pre-miRNA, presents a 5' phosphate and 3' hydroxy termini. The produced pre-miRNA is exported to the cytoplasm by Exportin-5/RnaGTP. Exp-5 forms a nuclear heterotrimer with RanGTP and pre-miRNA, resulted from Drosha processing. This interaction, which is dependent on RNA structure but independent of sequence, stabilizes the nuclear pre-miRNA and promotes the export to the cytoplasm.

It has been proposed that Drosha may recognize the primary precursor through the stem-loop structure and then cleave the stem at a fixed distance from the loop to liberate the pre-miRNA.

How the enzyme is capable to discriminate the pri-miRNA stem-loop structure in respect to the others stem-loop cellular RNAs is not clear, but probably proteins associated with Drosha confer specificity to this process. In fact, Drosha has been found as a part of large protein complex of ~650kDa, which is known as the “Microprocessor”, where Drosha interacts with its cofactor, the Di George syndrome critical region gene 8 (DGCR8) protein in human and Pasha in *Drosophila melanogaster* (9).

The Microprocessor appears to represent a heterotetramer consisting of two Drosha and two DGCR8 molecules; because DGCR8 contains two consensus dsRNA binding domain, this protein may play an important role in the substrate discrimination and binding.

The resulting product of cropping, the pre-miRNA, presents a 5' phosphate and 3' hydroxy termini, and two or three nucleotides single-stranded overhanging ends, classic characteristics of RNase III cleavage of dsRNAs. After the Microprocessor nuclear activity, the produced pre-miRNA is exported to the cytoplasm by Exportin-5/RnaGTP (10).

Exp-5 forms a nuclear heterotrimer with RanGTP and pre-miRNA, resulted from Drosha processing. This interaction, which is dependent on RNA structure but independent of sequence, stabilizes the nuclear pre-miRNA and promotes the export to the cytoplasm. In any export, once the Exp5-

RanGTP-pre-miRNA complex has reached the cytoplasm through the nuclear pore, the RnaGTP is hydrolyzed to RanGDP and the pre-miRNA is released.

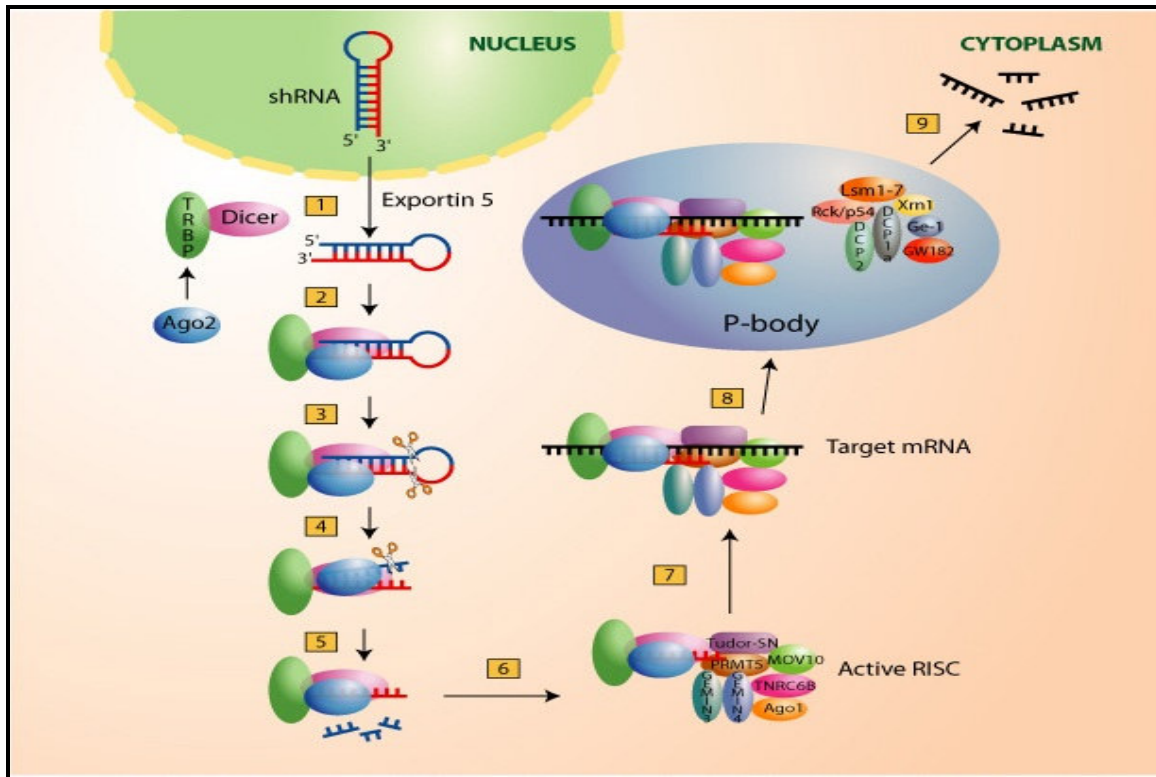
Arrived into the cytoplasm, the pre-miRNA is processed in 18~22 nucleotides miR duplexes by the cytoplasmic Rnase III Dicer and, in humans, its partner TRBP. The PAZ domain of Dicer is thought to interact with the nucleotides 3' overhang present in the pre-miRNA hairpin while the dsRNA binding domain binds the stem and defines the distance of cleavage from the base of pre-miRNA. The cleavage 22nt-long miRNA duplexes have a reduced half-life. Normally, one strand of this duplex is degraded whereas the other strand accumulates as a mature miRNA.

### **microRNA in action: RISC and gene target inhibition**

In the RNA duplex produced from the Dicer activity, the mature miRNA is only partially paired to the miRNA, the small RNA that resides on the opposite pre-miRNA stem. From the miRNA-miRNA duplex, only the miRNA enters preferentially in the protein effector complex, the RNA Induced Silencing Complex (RISC) or miRNAsC or miRgonaute, which mediates the degradation or translation inhibition of mRNAs target gene (Figure 2) (11).

Several proteins have identified as essential components of RISC, but only a few have been functionally characterized in the post-translational regulation. The core component of every RISC is a member of the Argonaute (Ago) protein family, whose members present a central PAZ domain like Dicer and a carboxy terminal PIWI domain. This domain binds the miR/miR duplex to

the 5' end whereas the PAZ domain binds to the 3' end of singled-strand RNAs; moreover, structural and biochemical studies have suggested that the



**Figure 2**

1-Plasmid-expressed short hairpin (shRNA) requires the activity of endogenous Exportin-5 for nuclear export (1).

2-Ago2 (Argonaute 2) is recruited by TRBP (2) that forms a dimer with Dicer (3) and then receives the shRNA (4-6).

3-The shRNA is cleaved in one step by Dicer generating a 19-23 nt duplex siRNA with 2 nt 3' overhangs.

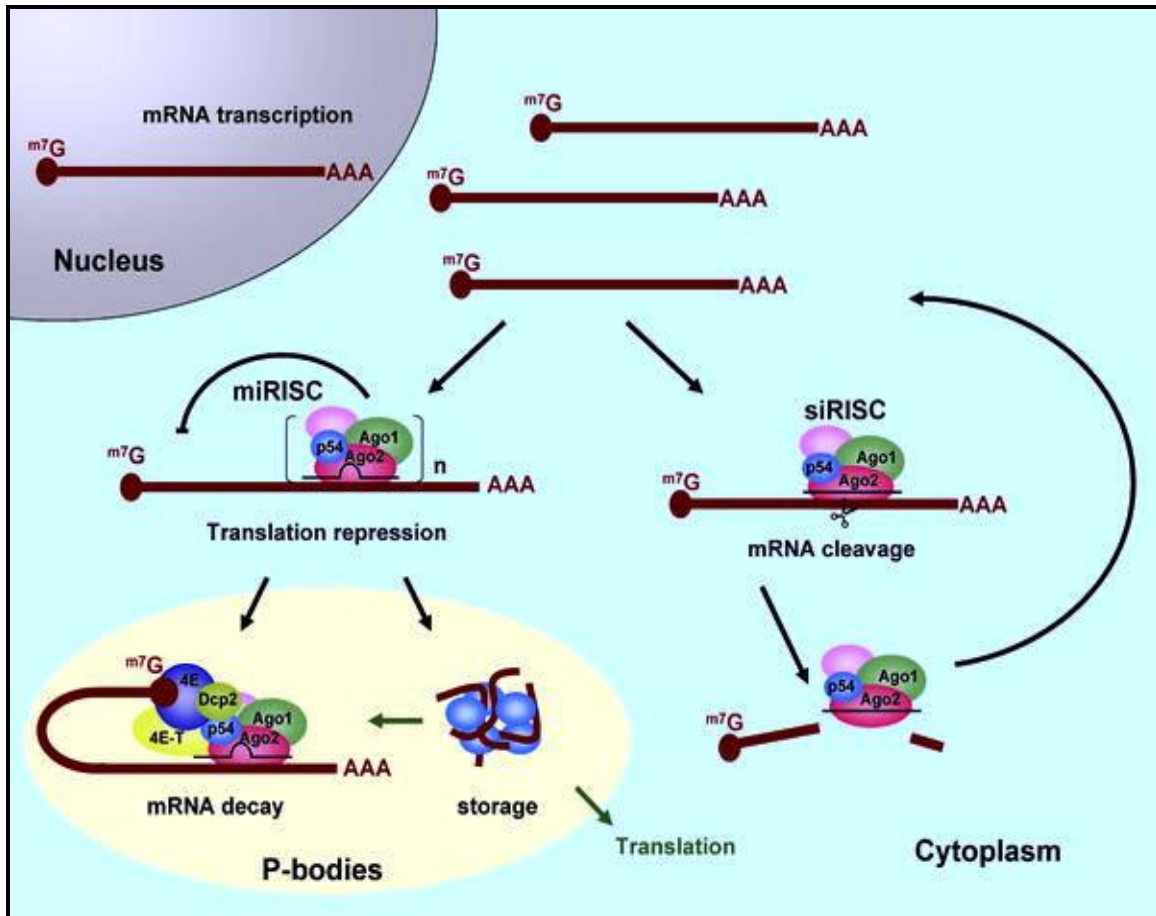
4-After identification of the "guide strand" in the siRNA duplex, the "passenger strand" is cleaved by Ago2 (4).

5-The "guide strand" is released.

6- The "guide strand" is integrated in the active RNA Interference Specificity Complex (RISC) that contains different argonautes and argonaute-associated proteins (7).

Ago proteins are the target-cleaving endonuclease of RISC and in this activity the complex is helped and coordinated by other proteins whose function is not really understood like RNA-binding protein VIG, the Fragile-X related protein in *Drosophila*, the exonuclease Tudor-SN and many other putative helicases (12).

In the human cells, after the microRNAs transfection by vectors or miRNA precursors, and the following activation of RISC activity, the core component of RISC, together with the triggering miRNA target mRNA, is concentrated in cytoplasmic foci known as Processing bodies (P-bodies) or GW-bodies. According with this triggered RISC localization, the researchers thought that the microRNAs, in association with AGO proteins, might be capable to repress the translation at ribosomal level and to re-localize mRNA targets to the P-bodies (Figure 3) (13).



**Figure 3**

Post-transcriptional processes have a central role in the regulation of eukaryotic gene expression. Although it has been known for a long time that these processes are functionally linked, often by the use of common protein factors, it has only recently become apparent that many of these processes are also physically connected. Indeed, proteins that are involved in mRNA degradation, translational repression, mRNA surveillance and RNA-mediated gene silencing, together with their mRNA targets, colocalize within discrete cytoplasmic domains known as P bodies. The available evidence indicates that P bodies are sites where mRNAs that are not being translated accumulate, the information carried by associated proteins and regulatory RNAs is integrated, and their fate — either translation, silencing or decay — is decided.

## **microRNAS: function in normal and disease states**

How do miRNAs work and what genes do they regulate? Expression profiling and functional studies suggest that miRNAs play a key role in biology, having been associated not only with physiological, but also with pathological processes such as cancer.

In lower species, miRs are involved in a variety of basic processes, e.g., cell proliferation and apoptosis (14, 15), neuronal development (16), fat metabolism (17) and stress response (18). In some studies, key target mRNAs have been identified but relatively little is known of the functional role of miRNAs in mammalian species. We do know, however, that miR-181 is involved in the control of lymphopoiesis (19) miR-375 regulates insulin secretion by targeting myotrophin mRNA (20), and the miR-let7 family may plays a role in oncogenesis via RAS oncogene mRNAs (21).

Furthermore, enforced expression of the miR-17-92 cluster from chromosome 13q32-33 in conjunction with c-myc accelerates tumor development in a mouse B-cell lymphoma model (22).

Two microRNAs from the same cluster, miR-17-5p and miR-20a negatively regulates the E2F1 transcription factor, a gene proved to function as a tumor suppressor in some experimental systems (23).

miR-15a and miR-16-1 are deleted or down-regulated in the majority of chronic lymphocytic leukemia (24). Functional studies indicated that miR-221&222 inhibit normal erythropoiesis and erythroleukemic cell growth at least in part via Kit receptor down-modulation, (25) and their ectopic overexpression directly results in p27<sup>Kip1</sup> down-regulation in aggressive prostate (26).

### **Apoptosis: extrinsic and intrinsic pathway**

Lung tumors are among the most deadly types of cancer. Advances in standard treatments for this tumor, such as surgery, radiotherapy, and chemotherapy, have not significantly increased patient survival. One of the most important issues that affects survival rate is resistance to therapeutic drugs. Only 20-30% of treated NSCLC patients have clinical evidence of a response. Therefore, the development of new therapeutic strategies is necessary for the treatment of this type of cancer.

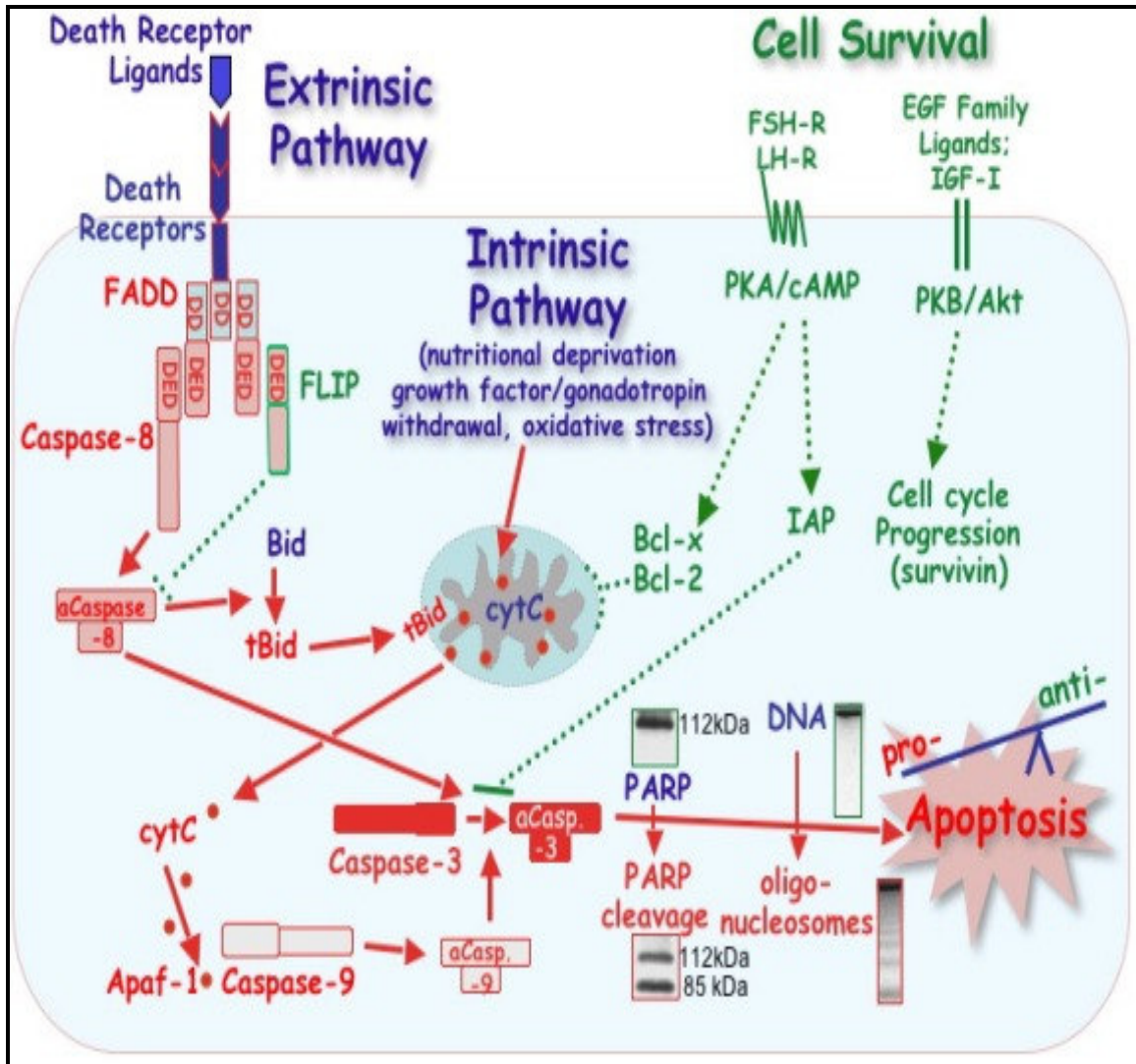
The Apo2L/TNF- $\alpha$ -related apoptosis-inducing ligand (TRAIL) is a relatively new member of the TNF family known to induce apoptosis in a variety of cancers (27). Apoptosis represents a tightly regulated and evolutionarily conserved program of cell suicide which is involved in normal cellular homeostasis (28).

Most chemotherapeutic drugs kill cancer cells by inducing apoptosis, and many similarities exist in cellular response to drug-induced apoptosis, regardless of their primary target (14, 29-37). Apoptosis, from the Greek word for “falling off ” or “dropping off” (as leaves from a tree), is defined by distinct morphological and biochemical changes mediated by a family of cysteine aspartic acid-specific proteases (caspases), which are expressed as inactive precursors or zymogens (pro-caspases) and are proteolytically processed to an active state following an apoptotic stimulus. To date, approximately 14 mammalian caspases have been identified and can be roughly divided into three functional groups: apoptosis initiator (including caspase-2, -9, -8, -10), apoptosis effector (including caspase-3, -6, -7), and cytokine maturation (including caspase-1,-4,-5,-11,-12,-13,-14)(38-40). . Two separable pathways lead to caspases activation: the extrinsic pathway



and the intrinsic pathway (Figure 2). The extrinsic pathway is initiated by ligation of transmembrane death receptors (Fas, TNF receptor, and TRAIL receptor) with their respective ligands (FasL, TNF, and TRAIL) to activate membrane-proximal caspases (caspase-8 and –10), which in turn cleave and activate effector caspases such as caspase-3 and –7.

The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins, such as cytochrome c. Cytochrome c, released from the mitochondrial intermembrane space to cytoplasm, works together with the other two cytosolic protein factors, Apaf-1 (apoptotic protease activating factor-1) and procaspase-9, to promote the assembly of a caspase-activating complex termed the apoptosome, which in return induces activation of caspase-9 and thereby initiates the apoptotic caspase cascade (39-43).



**Figure 4**

Known as the "death receptor pathway" the extrinsic or caspase 8/10 dependent pathway is activated by ligand binding. The "death receptors" are specialized cell-surface receptors including Fas/CD95, tumor necrosis factor-alpha (TNF-alpha) receptor 1, and two receptors, DR4 and DR5, that bind to the TNF-alpha related apoptosis-inducing ligand (TRAIL). The extrinsic and intrinsic pathways unite in the activation of Caspase-3, though the two pathways communicate through the pro-apoptotic Bcl-2 family member Bid before uniting at the shared activation of Caspase-3.

The primary regulatory step for mitochondrial-mediated caspase activation (the intrinsic pathway) might be at the level of cytochrome *c* release (42, 43). The known regulators of cytochrome *c* release are Bcl-2 family proteins (41-45).

According to their function in apoptosis, the mammalian Bcl-2 family can be divided into pro-apoptotic and anti-apoptotic members. The pro-apoptotic members include Bax, Bcl-Xs, Bak, Bok/Mtd, which contain 2 or 3 Bcl-2 homology (BH) regions, and molecules such as Bad, Bik/Nbk, Bid, Hrk/DP5, Bim/Bod, and Blk, which contains only the BH3 region. The anti-apoptotic Bcl-2 family members include Bcl-2, Bcl-XL, Bcl-w, A1/Bfl-1, Mcl-1, and Boo/Diva, which contain three or four regions with extensive aminoacid sequence similarity to Bcl-2 (BH1-BH4) (45). Overexpression of the anti-apoptotic molecules such as Bcl-2 or Bcl-XL blocks cytochrome *c* release in response to a variety of apoptotic stimuli. On the contrary, the pro-apoptotic members of the Bcl-2 family proteins (such as Bax and Bid) promote cytochrome *c* release from the mitochondria. Pro-apoptotic and anti-apoptotic members of the Bcl-2 protein family can physically interact. For example, binding of BH-3 only proteins (e.g., Noxa, Puma, Bad, and Bim) to anti-apoptotic Bcl-2 proteins (e.g., Bcl-2 and Bcl-XL) results in activation of Bax and Bak (44, 45). In addition, there is considerable cross-talk between the extrinsic and intrinsic pathways. For example, caspase-8 can proteolytically activate Bid, which can then facilitate the release of cytochrome *c* and amplifies the apoptotic signal following death receptor activation. (36, 39-43).

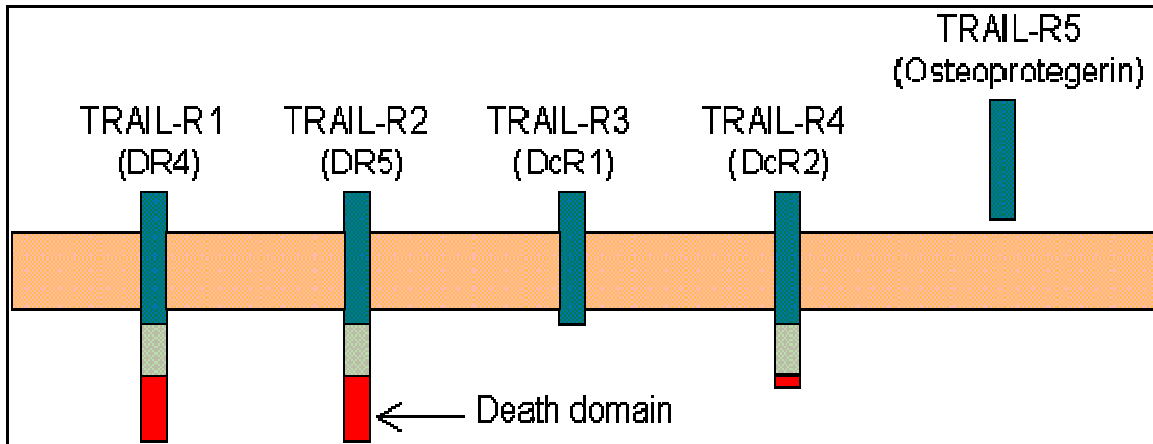
## **TRAIL and miRs**

Most of the anticancer agents either directly induce DNA damage or indirectly induce secondary stress-responsive signaling pathways to trigger apoptosis by activation of the intrinsic apoptotic pathway, and some can simultaneously activate the extrinsic receptor pathway. Therefore, molecules or signaling events that regulate the processes of apoptosis can also affect cellular response to drugs.

TRAIL (TNF-related apoptosis-inducing ligand, also called APO-2) is a protein consisting of 281 amino acids that can bind to five receptors (Fig. 5) of which four are located at the cell surface: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2). Only two of these receptors, R1 and R2, contain a functional cytoplasmic death domain motif and are capable of delivering the apoptotic signal of TRAIL by association of the death domain with the Fas-associated death domain protein (FADD), containing the death effector domain, which is involved in the activation of caspase-8 (46).

The other two receptors, DcR1 and DcR2, are “decoy receptors” and lack the ability to initiate the apoptotic cascade.

Treatment with TRAIL induces programmed cell death in a wide range of transformed cells, both in vitro and in vivo, without producing significant effects in normal cells (27, 46). This unique property makes TRAIL an attractive candidate for cancer therapy. Preclinical experiments in mice and nonhuman primates have shown that administration of TRAIL suppresses tumor growth without apparent systemic cytotoxicity (47, 48). Therefore, TRAIL represents a promising anti-cancer agent.



**Figure 5**

Schematic drawing of TRAIL receptors, which can be divided into two categories:

**Death receptors:** TRAIL-R1 and TRAIL-R2 contain the death domain, capable of delivering the apoptotic signal of TRAIL by association of the death domain with FADD, containing the death effector domain, which is involved in the activation of caspase-8

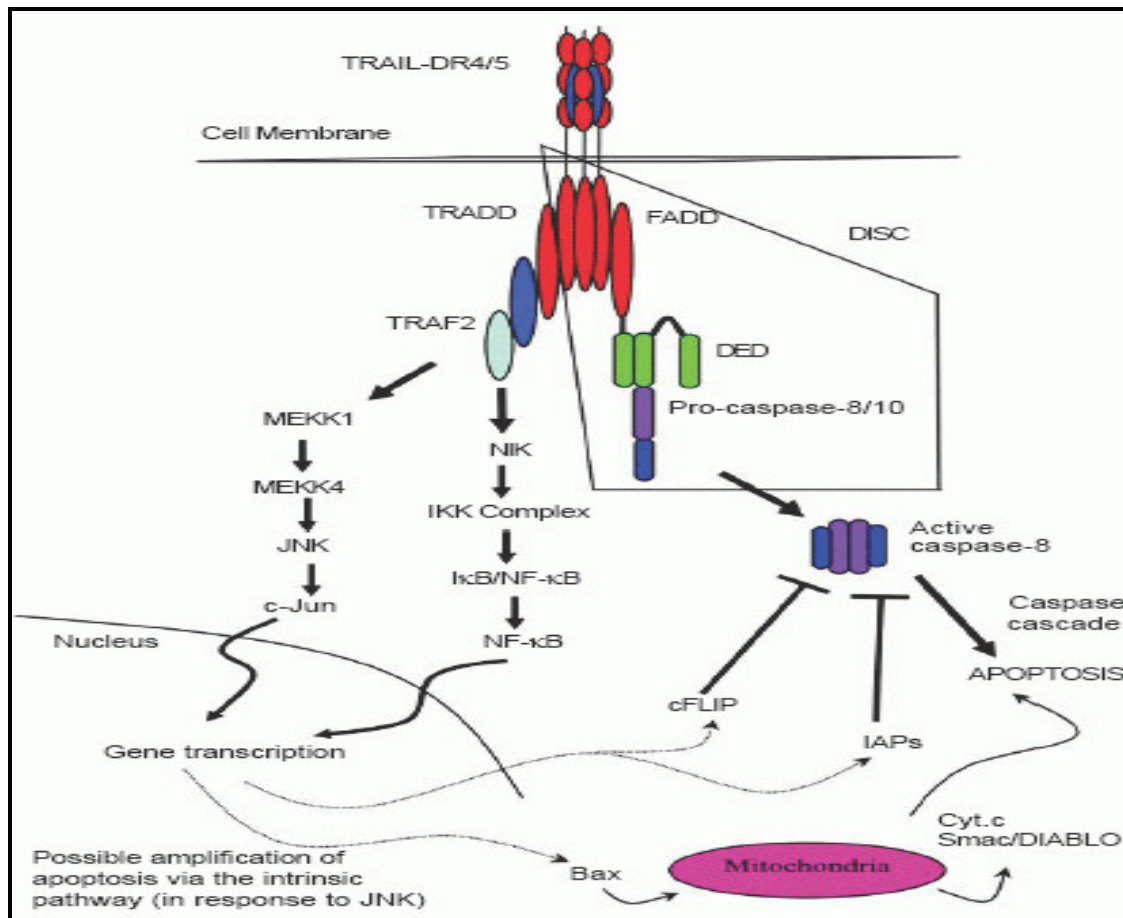
**Decoy receptors:** TRAIL-R3 and TRAIL-R5 lack the death domain while TRAIL-R4 contains a truncated non-functional death domain. These three receptors can bind to TRAIL, but cannot induce apoptosis. TRAIL-R5 is secreted to the extracellular fluid. All other receptors are transmembrane proteins.

However, a significant proportion of human cancer cells are resistant to TRAIL-induced apoptosis, and the mechanism of sensitization seems to differ among cell types.

Different studies relate resistance to TRAIL-induced cell death to downstream factors. It has been shown that down-regulation of PED or cellular FLICE-like inhibitory protein (c-Flip) can sensitize cells to TRAIL-induced apoptosis (47, 48). However the mechanism of TRAIL resistance is still largely unknown.

In this study, in order to identify novel mechanisms implicated in TRAIL resistance, we performed a genome-wide expression profiling of miRs in four different cell lines in human non small cell lung cancer (NSCLC).

We found that miR-221&222 are markedly up-regulated in TRAIL-resistant, and down-regulated in TRAIL-sensitive, NSCLC cells. Our experiments indicate that miR-221&222 modulate TRAIL sensitivity in lung cancer cells mainly by modulating p27<sup>kip1</sup> expression and TRAIL-induced caspase machinery.



**Figure 6**

Members of the tumor necrosis factor (TNF) super family of receptors induce apoptosis by recruiting adaptor molecules through death domain interactions.

The central adaptor molecule for these receptors is the death domain-containing protein FADD. FADD binds a death domain on a receptor or additional adaptor and recruits caspases to the activated receptor. FADD, caspases-8 and caspase-10 are recruited to the DISC, “death-inducing signalling complex”. DISC formation leads to activation of a protease cascade, finally resulting in cell death. The TRAIL death receptor-mediated “extrinsic” pathway and the “intrinsic” pathway, which is controlled by the interaction of members of the Bcl-2 family, interact with each other in the decision about life or death of a cell. Apoptotic and non-apoptotic signalling is influenced by the NF-κB, PKB/Akt and the MAPK signalling pathways.

The control of cell survival and death is through NF-κB–JNK cross-talk. Positive feedback loops exist between caspases and JNK. Negative feedback loops exist between NF-κB and caspases. NF-κB functions as a pro-survival transcription factor by inducing the expression of antiapoptotic genes, such as the Bcl-2 family members and caspase inhibitors. Activation of NF-κB also results in inhibition of prolonged JNK activation, mostly through inhibition of ROS accumulation.

## Experimental Procedures

**Materials-** Media, sera and antibiotics for cell culture were from Life Technologies, Inc. (Grand Island, NY, USA). Protein electrophoresis reagents were from Bio-Rad (Richmond, VA, USA) and Western blotting and ECL reagents from GE Health care. All other chemicals were from Sigma (St. Louis, MO, USA).

**Cell culture-** Human CALU-1 and A549 NSCLC cell lines were grown in DMEM containing 10% heat-inactivated FBS and with 2mM L-glutamine and 100U/ml penicillin-streptomycin. H460 and A459 cell lines were grown in RPMI containing 10% heat-inactivated FBS and with 2mM L-glutamine and 100U/ml penicillin-streptomycin.

**Western blotting-** Total proteins from CALU-1, A459, A549 and H460 cells were extracted with RIPA buffer (0,15mM NaCl, 0,05mM Tris-HCl, pH 7,5, 1% Triton, 0,1% SDS, 0.1% sodium deoxycolate and 1% Nonidet P40).

Fifty µg of sample extract were resolved on 7.5-12% SDS-polyacrylamide gels using a mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA) and transferred to Hybond-C extra nitrocellulose. Membranes were blocked for 1hr with 5% non-fat dry milk in TBS containing 0.05% Tween-20, incubated for 2h with primary antibody, washed and incubated with secondary antibody, and visualized by chemiluminescence. The following primary antibodies were used: Kit (R&D System), and a secondary anti-goat IgG



antibody peroxidase conjugate (Chemicon); anti-TRAIL-R1, -R2, -R3, and -R4 (Santa Cruz, Inc.), anti-p27<sup>kip1</sup> (Santa Cruz, Inc), anti-caspase 8 (Cell Signaling), anti-caspase 3 and anti-PARP (Santa Cruz, Inc), anti- $\beta$ -actin antibody (Sigma). Expression levels were analyzed with SCION IMAGE.

**FACS analysis-** For flow cytometry analysis of cell surface Kit, cells were stained with primary anti-human h-SCFR affinity purified goat IgG or mouse MAb 002 (isotype control) (both from R&D Systems), followed by secondary antibody, Fluorescein conjugated goat F(ab')<sub>2</sub> (R&D Systems).

**Cell death and cell proliferation quantification-** Cells were plated in 96-well plates in triplicate and incubated at 37°C in a 5% CO<sub>2</sub> incubator. Super-Killer TRAIL (Alexis Biochemicals, Lausen, Switzerland) was used for 24-48 hrs at 400 ng/ml. Cell viability was evaluated with the CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer's protocol. Metabolically active cells were detected by adding 20  $\mu$ l of MTS to each well. After 2 h of incubation, the plates were analyzed in a Multilabel Counter (Bio-Rad, Richmond, VA, USA). Apoptosis was assessed using annexin V-FITC Apoptosis Detection Kits followed by flow cytometric analysis. Cells were seeded at  $1.8 \times 10^6$  cells per 100-mm dish, grown overnight in 10% FBS/RPMI, washed with PBS, then treated for 24 hours with 200 ng TRAIL. Following incubation, cells were washed with cold PBS and removed from the plates by trypsinization. The resuspended cells were washed with cold PBS and stained with FITC-conjugated annexin V antibody and propidium iodide (PI) according to the instructions provided by the manufacturer (Roche Applied Science,

Indianapolis, IN). Cells (50,000 per sample) were then subjected to flow cytometric analysis. The fraction of H460 cells treated with TRAIL was taken as the apoptotic cell population. The percentage of apoptosis indicated was corrected for background levels found in the corresponding untreated controls.

**RNA extraction, Northern blotting-** Total RNA was extracted with TRIzol solution (Invitrogen) and the integrity of RNA was assessed with an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA). Northern blotting was performed as described in ref (4). Ten micrograms of total RNA from cell lines were loaded onto a precast 15% denaturing polyacrylamide gel (Bio-Rad). The RNA was then electrophoretically transferred to bright-Star blotting membranes (Ambion). The oligonucleotides used as probes were the complementary sequences of the mature miRNA (miRNA registry): miR221, 5'-GAAACCCAGCAGACAATGTAGCT-3'; miR222, 5'-GAGACCCAGTAGCCAGATGTAGCT-3'. miR probes were end-labeled with [ $\gamma$ -<sup>32</sup>P]-ATP by T4 polynucleotide kinase (USB, Cleveland). Prehybridization and hybridization were carried out in Ultrahyb Oligo solution (Ambion) containing 10<sup>6</sup> cpm/ml probes overnight 37°C. The most stringent wash was with 2X SSC and 1% SDS at 37°C. For reuse, blots were stripped by boiling and reprobed. As a loading control U6 rRNA was used. The image of Northern hybridization signals was produced by using STORMSCANNER and IMAGEQUANT TL software (Molecular Dynamics)

**miRNA Microarray experiments-** 5 µg of total RNA from each sample was reverse transcribed using biotin end labeled random-octamer oligonucleotide primer.

Hybridization of biotinlabeled complementary DNA was performed on a new Ohio State University custom miRNA microarray chip (OSU\_CCC version 3.0), which contains 1150 miRNA probes, including 326 human and 249 mouse miRNA genes, spotted in duplicates. The hybridized chips were washed and processed to detect biotin-containing transcripts by streptavidin-Alexa647 conjugate and scanned on an Axon 4000B microarray scanner (Axon Instruments, Sunnyvale, Calif ).

Raw data were normalized and analyzed in GENESPRING 7,2 software (zcomSilicon Genetics, Redwood City, CA). Expression data were median-centered by using both the GENESPRING normalization option and the global median normalization of the BIOCONDUCTOR package ([www.bioconductor.org](http://www.bioconductor.org)) with similar results. Statistical comparisons were done by using the GENESPRING ANOVA tool, predictive analysis of microarray (PAM) and the significance analysis of microarray (SAM) software (<http://www-stat.stanford.edu/~tibs/SAM/index.html>).

**Real Time PCR-** Real-time PCR was performed using a standard TaqMan PCR Kit protocol on an Applied Biosystems 7900HT Sequence Detection System (P/N: 4329002, Applied Biosystems). The 10 µl PCR reaction included 0.67 µl RT product, 1 µl TaqMan Universal PCR Master Mix (P/N: 4324018, Applied Biosystems), 0.2 mM TaqMan probe, 1.5 mM forward primer and 0.7 mM reverse primer. The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and

60 °C for 1 min. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The comparative C<sub>T</sub> method for relative quantization of gene expression (User Bulletin #2, Applied Biosystems) was used to determine miRNA expression levels. Experiments were carried out in triplicate for each data point, and data analysis was performed by using software (BioRad). To normalize the expression levels of target genes, U6 has been used.

**Bioinformatics-** miR target prediction of the differentially expressed miRs was performed with TARGETSCAN, MIRANDA and PICTAR software.

**Pre-miRs and anti-miR miRNA inhibitors transfection in NSCLC cells-** TRAIL sensitive cell lines (H460) were cultured to 80% confluence in p60 plates with a serum-free medium without antibiotics and then transfected with 100 nmol of pre-miR-221 and 222 oligonucleotides or Negative Control for 48 hrs (Ambion). CALU-1 cells were cultured to 80% confluence in p60 plates with a serum-free medium without antibiotics. 100 nmol of -221&222 anti miR miRNA inhibitors (Ambion) were transiently transfected in cells using LIPOFECTAMINE 2000 according to manufacturer's instructions. Cells were incubated in the presence of the specific anti-miR miRNA inhibitors and Negative Control for 48 hrs.

The Anti-miR miRNA Inhibitor Negative Control is a random sequence anti-miR molecule offered for use as a negative experimental control. The Anti-miR Negative Control has been extensively tested in many human cell

lines and tissues and validated to not produce any identifiable effect on known miRNAs function.

Subsequently, TRAIL-induced cell death was analyzed as previously described.

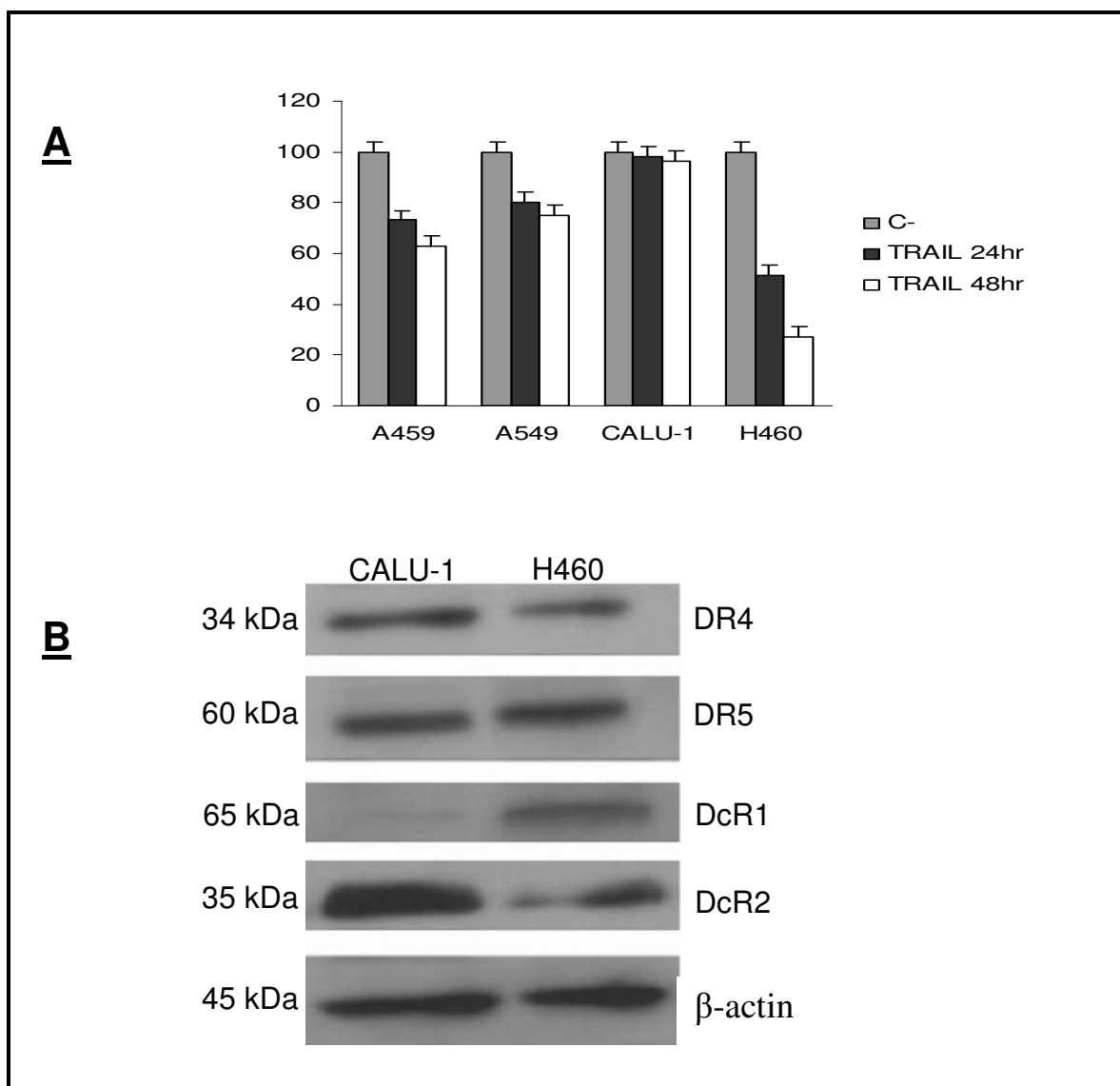
**DNA demethylating agents and HDACis-** TRAIL sensitive H460 cell line was treated with different concentrations of Trichostatin A (TSA) (100, 200, 300, and 500 nM), sodium butyrate (10, 25, 50, and 75 mM) or 5'-Aza-2'-deoxycytidine (1, 5, 10 mM). Moreover, we performed a time course with 10mM 5'-Aza and 300nM TSA. Northern blotting were performed as previously described.

**Anti-p27<sup>Kip1</sup> siRNA transfection in NSCLC-** H460 cells were cultured to 80% confluence and transiently transfected using LIPOFECTAMINE 2000 with anti-p27<sup>Kip1</sup> siRNA (Santa Cruz) and anti- Kit siRNA (Dharmacon), a pool of 4 target specific 20-25 nt siRNAs designed to knock down gene expression. 20 nmol of p27<sup>Kip1</sup> RNA was transfected with 6µl transfection reagent, as described in the manufacturer's protocol.

## RESULTS

### **The cytotoxic effects of TRAIL in human non small cell lung cancer (NSCLC)-**

We analyzed TRAIL sensitivity of different human NSCLC cell lines: A459, A549, CALU-1 and H460. Cells were exposed to TRAIL for 24 and 48 hours (Figure 7A) after which cell death was assessed using an MTT assay or by FACS with annexin V and propidium iodide staining (data not shown). As shown in Figure 7A, H460 cells underwent TRAIL-induced cell death whereas CALU-1 cells did not display sensitivity when exposed to soluble TRAIL; A459 and A549 cells showed an intermediate sensitivity. A possible mechanism of the differential sensitivity of the tested cells to TRAIL-induced apoptosis could be due to the variability of the cell surface levels of the death and decoy receptors resulting in increased apoptotic signaling in the sensitive cells. However, TRAIL receptor isoforms analyzed by western blot (Figure 7B) or FACS analysis (data not shown) revealed comparable levels of expression. Furthermore, although H460 cells do not express DcR1, the expression of DcR2 receptor is greater than CALU-1. Therefore, the expression of the decoy receptors within the two cell lines is balanced.



**Figure 7**

(A) In order to assess TRAIL sensitivity, NSCLC cells were incubated with Super-Killer-TRAIL (400ng/ml) for 24 and 48 hours and viability evaluated as described in the methods section. Experiments were repeated four times in triplicate. H460 cells were more sensitive to TRAIL-induced apoptosis compared to CALU-1. A459 and A549 exhibited an intermediate sensitivity. (B) TRAIL receptors expression in CALU-1 and H460 cells. Fifty micrograms of total extract was loaded onto 10% SDS-PAGE. The membrane was blotted with anti DR4, DR5 (1μg/ml) and DcR1 and DcR2 antibody (0.5 μg/ml). Loading control was obtained with anti-β actin antibody (1:5000).

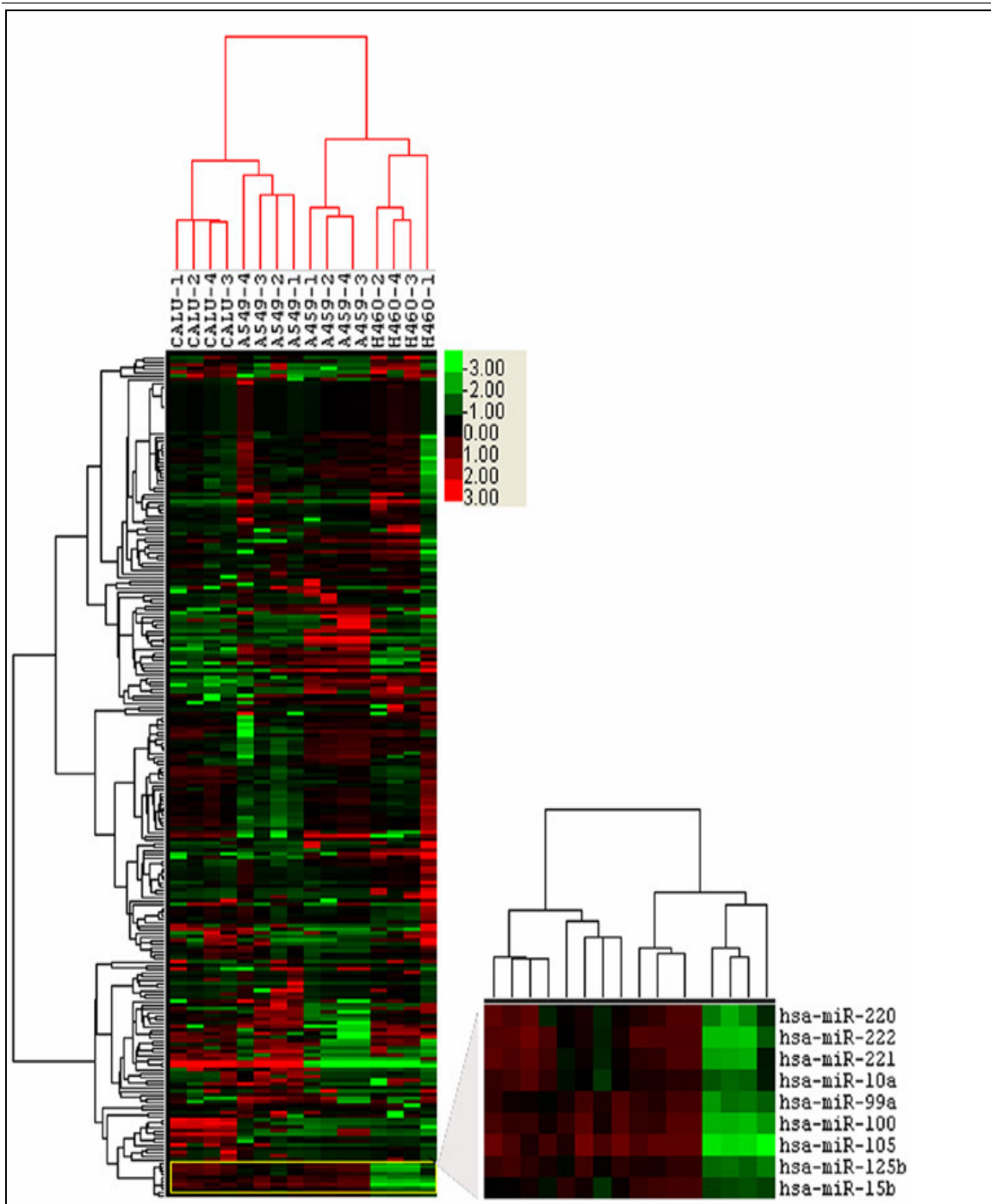
### **miRs expression screening in TRAIL resistant vs sensitive NSCLC cell lines-**

To investigate the involvement of miRs in TRAIL resistance, we analyzed the miRs expression profile in TRAIL-resistant (CALU-1) and semi-resistant NSCLC cell lines (A459, A549) versus TRAIL sensitive cell line (H460). The analysis was performed with a microarray chip containing 1150 miR probes, including 326 human and 249 mouse miRs, spotted in duplicates (49). Pairwise significance analysis (PAM) of the microarray indicated that five miR genes were significantly overexpressed in resistant NSCLC cells with a >1.5-fold change (Figure 8). These miRs were: miR-222, miR-100, miR-221, miR-125b, miR-15b (Table 1). Three of these miRs, miR-222, miR-221, and miR-100, showed dramatic overexpression with 5- to 8-fold higher levels in resistant NSCLC cells compared with the sensitive NSCLC cells. Down-regulation occurred for only two miRs, miR-9 and miR-96.

To validate the microarray analysis, we performed quantitative Real-Time-polymerase chain reaction (qRT-PCR) of the most overexpressed miRs (miR-222, miR-100, miR-221, miR-125b) and of down-regulated miR-9 in CALU-1 TRAIL-resistant cells, A459 and A549 semi-resistant cells and H460 TRAIL-sensitive cells. The analysis confirmed the results obtained by the microarray (Figure 9A).

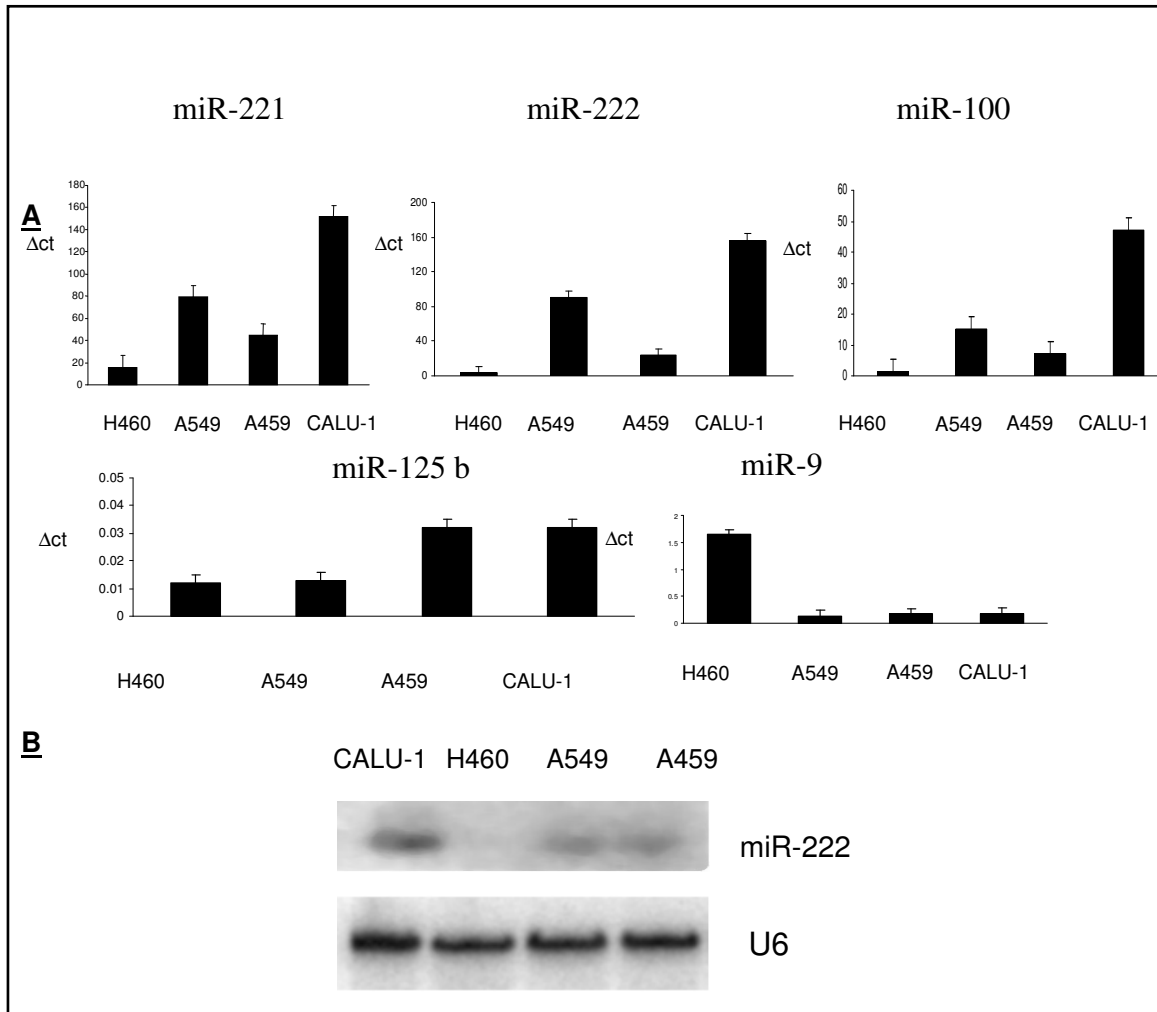
The expression of miR-222 in NSCLC cells was confirmed also with Northern blot (Figure 9B).





**Figure 8**

Fold changes (resistant vs. sensitive) of the miRs present in NSCLC cells. The tree displays the log<sub>2</sub> transformation of the average fold changes. Arrays were mean centered and normalized by using GENE CLUSTER 2.0. Average linkage clustering was performed by using uncensored correlation metric.



**Figure 9**

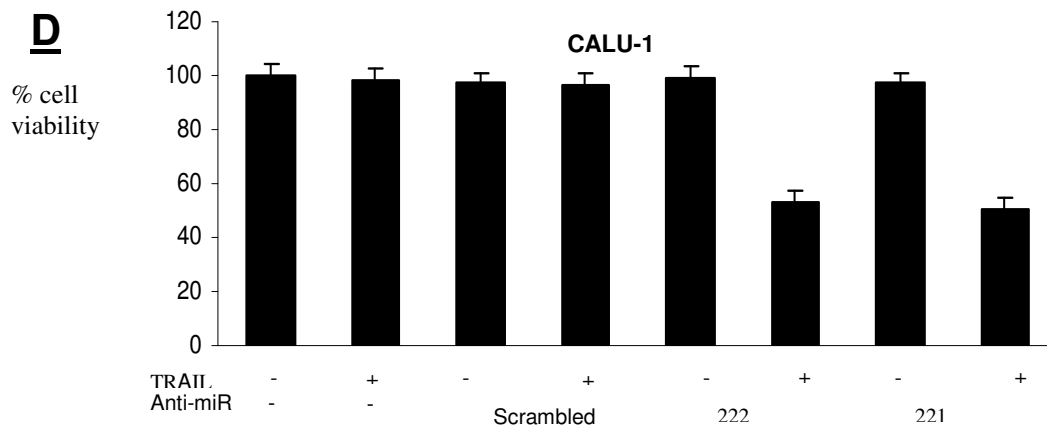
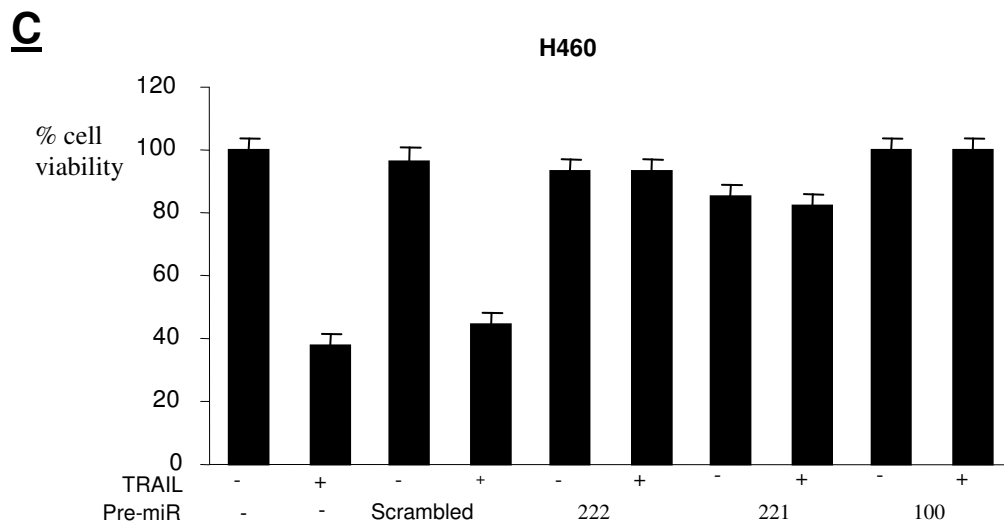
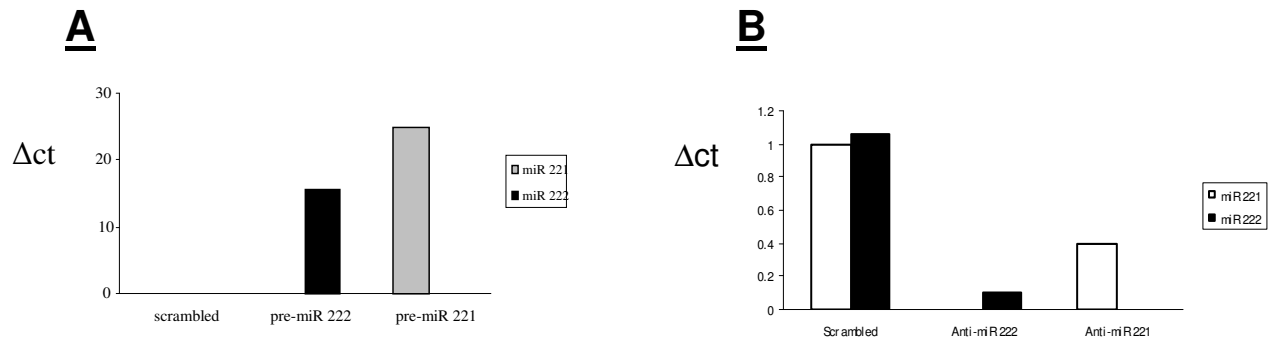
(A) Real-time PCR of the most promising miRNAs targets obtained with microarray screening was performed by extracting RNA from the different NSCLC cells, as described in the methods section. 5  $\mu\text{g}$  of RNA in 10  $\mu\text{l}$  PCR reaction was used. TaqMan  $\Delta\text{ct}$  values were converted into absolute copy numbers using a standard curve from synthetic lin-4 miRNA. miRs 222, 100, 221, 125b were markedly up-modulated in resistant but not in sensitive cell cultures, while miR-9 was markedly down-modulated.

(B) Northern blot analysis of miR-222 expression. Ten micrograms of RNA were loaded onto a precast 15% denaturing polyacrylamide gel (Bio-Rad). RNA was then electrophoretically transferred to bright-Star blotting membranes and membrane incubated with labelled miR-222 probe. miR-222 was strongly up-regulated in TRAIL-resistant CALU-1 cells.

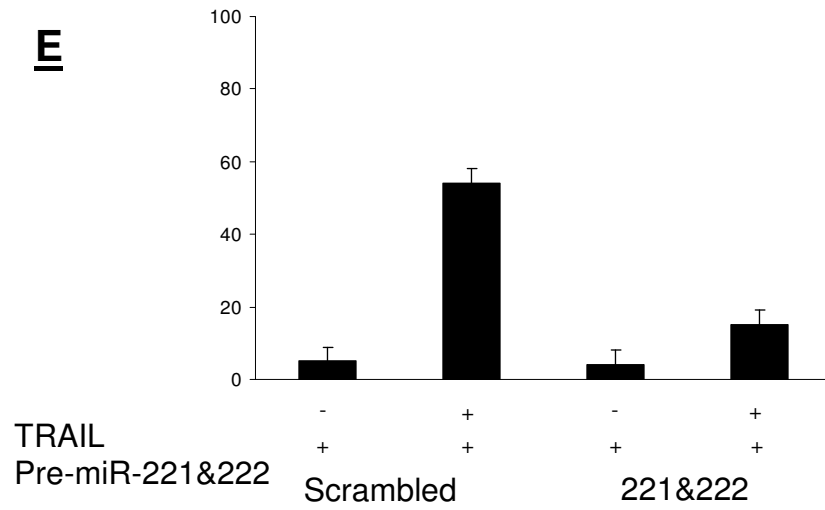
### **Role of miR-221 and 222 in TRAIL resistance in NSCLC-**

In order to test the role of these overexpressed miRs in TRAIL sensitivity in lung cancer, we transfected H460 TRAIL-sensitive cells with pre-miR-221 and -222. Increased expression of these miRs upon transfection was confirmed by Real-Time PCR (Figure 10A). Overexpression of miR-221 and -222 in H460 cells made these cells more resistant to TRAIL-induced cell death by about 40% (Figure 10C). Interestingly, miR-100 also increased TRAIL resistance, while pre-miR-125b and control miR overexpression did not produce any effect (data not shown). A propidium iodide staining in H460 transfected with both pre-miR-221 and 222 confirmed these results (Figure 10E).

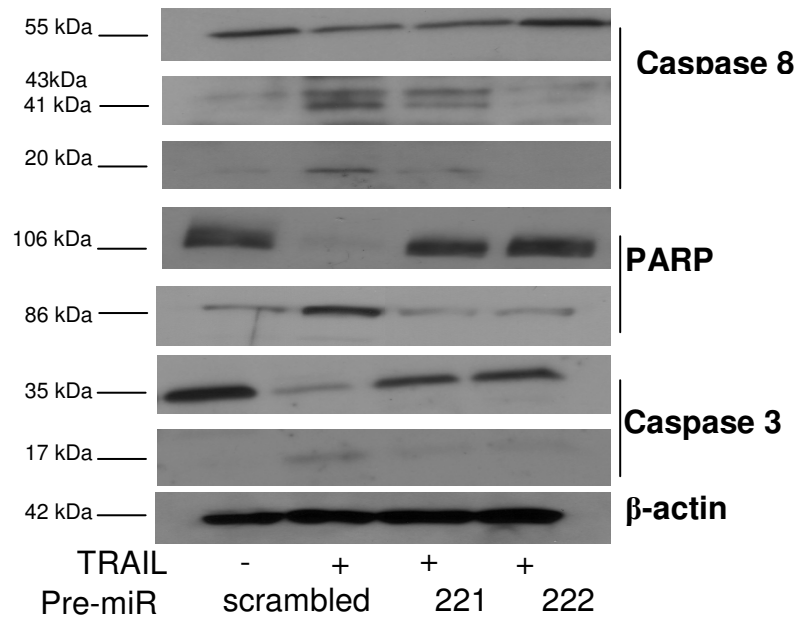
Anti-miR inhibitors are sequence-specific and chemically modified to specifically target and knock down individual miR molecules. We transfected CALU-1 TRAIL-resistant cells with anti-miR inhibitor -221 and -222 and then assessed TRAIL sensitivity. As shown in Figure 10B, the levels of miR-221 and -222, assessed by RT-PCR, were reduced. Interestingly, the inhibition of miR-221 and 222 expression with the specific anti-miR inhibitor, was able to change the insensitive TRAIL phenotype to a sensitive one (Figure 10D). A scrambled non-specific anti-miR did not produce any effect. We also tested the effects of miRs 221 and 222 on the activation of caspase 8, 3 and PARP. Interestingly, while in H460 cells, TRAIL induced the activation of caspase cascade, as assessed by the appearance of the cleaved fragment, the co-incubation of TRAIL with 222&221 premiR, induced a reduction of TRAIL-mediated cell death machinery activation (Figure 10F). TRAIL receptors expression was not affected by miR-221 and -222 up or down modulation (Figure 10G).

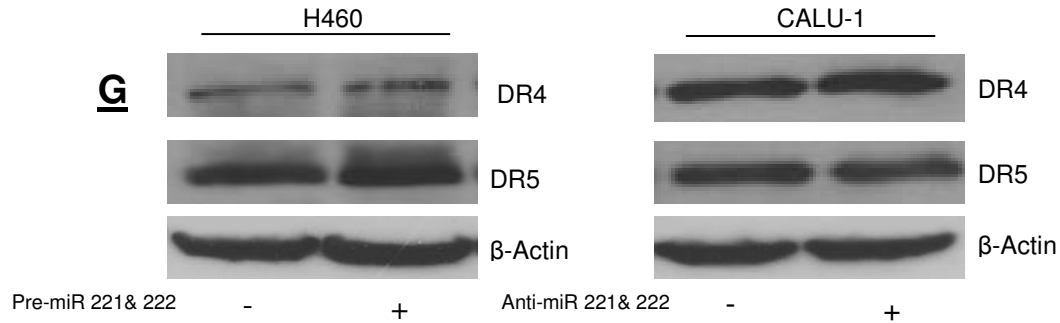


**E**



**F**





**Figure 10**

(A) Real time PCR analysis after transfection of pre-miR221&222 in H460 cells. RNA was extracted and analyzed as previously described. miR transfection resulted in an increase of miR-221 and -222 expression in H460 cells. (B) Real time-PCR analysis of miR-221&222 transfection in CALU-1 cells. Cells were transfected with 100 nmol of -221 or -222 anti-miR oligonucleotides. RNA was extracted and analyzed as previously described. Anti-miR transfection resulted in a decrease of miR-221&222 expression. (C) Cells were transfected either with control scrambled miR or with 100 nmol of pre-miR-221,-222 and -100. 24 hrs after transfection, cells were spitted into 96 well-plates and treated with 400 ng/ml of Super-Killer TRAIL for 24 hours. Cell viability was evaluated with the CellTiter Assay. miR-221&222 and miR-100 overexpression induced TRAIL resistance in H460 cells. (D) Effects of anti-miRs on cell death. Cells were transfected with 100nM of anti-miRs 222, 221 or a scrambled control. 24 hrs after transfection, cells were split into 96 well and treated with 400 ng/ml of Super-Killer TRAIL for 24 hrs, and cell viability was assessed as previously described. Down-regulation of miRs 221&222 in CALU-1 cells increased TRAIL sensitivity. (E) Propidium iodide (PI) staining of apoptotic cells- TRAIL was added for 24 hours and cell death was evaluated by PI staining and FACS analysis. miR221&222 overexpression induced TRAIL resistance in H460 cells. (F) The same cells (H460) were also assessed for caspase activation by Western blot. Eighty micrograms of total extract was loaded onto 10% SDS-PAGE. The membrane was blotted with anti caspase 8 (1:1000), PARP (1:200), or caspase 3 (1:200) antibodies. Loading control was obtained using anti-β actin antibody. (G) Western blot anti-DR4 and DR5 after miRs transfection. H460 cells were transfected with 100nM of -221 and -222 while CALU cells were transfected with anti miR-221 and -222. Fifty micrograms of total extract was loaded onto 10% SDS-PAGE. The membrane was blotted with anti DR4, DR5 (1μg/ml) and DcR1 and DcR2 antibody (0.5 μg/ml). Loading control was obtained with anti-β actin antibody (1:5000).

### **miR-221 and -222 expression is repressed in TRAIL sensitive cells by epigenetic modifications-**

In disease, recent studies have shown that miRNA expression profiles are distinct between normal tissues and derived tumors (50) and between different tumor types (49). Interestingly, down-regulation of subsets of miRNAs is a common finding in many of these studies (50, 51), suggesting that some of these miRNAs may act as putative tumor suppressor genes. This last indication has been studied in more detail for particular cases, and for example, the down-regulated let-7, miR-15/miR-16, and miR-127 target the oncogenic factors RAS, BCL-2, and BCL-6, respectively (21, 52, 53). One explanation is a failure at the posttranscriptional regulation of these miRNAs in cancer cells (54). However, additional mechanisms could also be invoked. Because the down-regulation of many relevant tumor suppressor genes in human cancer, such as hMLH1, BRCA1, and p16INK4a, has been tightly linked to the presence of CpG island promoter hypermethylation (55-57), we wondered if the same mechanism could be playing a role in the described loss of miRNA expression in tumors. In this regard, restoration of miRNA-127 expression in cancer cells by treatment with a DNA-demethylating agent has been recently reported (53). These recent and exciting data support the idea of an aberrant DNA methylation pattern of miRNA genomic loci in human tumors.

Histone deacetylase inhibitors (HDACis) are a promising new class of antineoplastic agents with the capacity to induce growth arrest and/or apoptosis of cancer cells. Furthermore, the down regulation of many relevant tumor suppressor genes in human cancer has been tightly linked to the presence of CpG island promoter hypermethylation (55, 57). Therefore, we

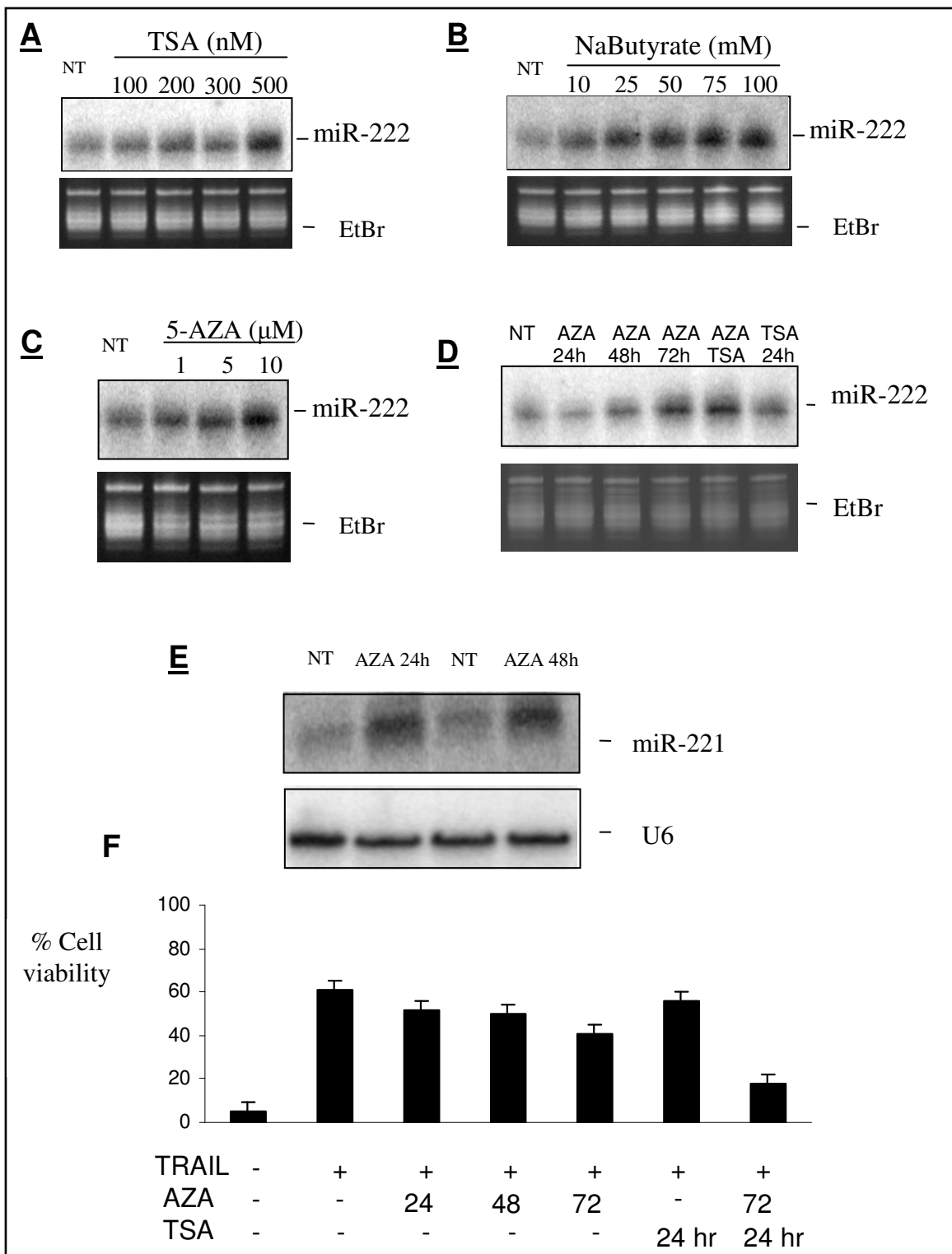
wondered whether the same mechanism could be playing a role in the loss of miR-221 and -222 expression in NSCLC cells.

We treated TRAIL sensitive H460 cell line for 24 hrs with different concentrations of Trichostatin A (TSA), sodium butyrate or the DNA demethylating agent, 5' aza-cytidine (Aza), and evaluated miR-222 levels by northern blot (Figure 11 A-D). Interestingly, we observed a dose and time-dependent effect of Aza, alone and combined with TSA treatment in the regulation of miR-222 expression levels. A comparable effect of HDACis or Aza was also observed in the regulation of miR-221 (Figure 11E).

Based on the findings described above, we tested the effects of epigenetic modifications on TRAIL sensitivity in H460 cells. As shown in Figure 11F we found, that the sensitivity of H460 cells to TRAIL was reduced upon HDACi or Aza treatment, in accordance with the increase of miR-221 and -222 expression. This effect was time-dependent and reached maximum level after coincubation with Aza (72hrs) and TSA (24 hrs).

Our results suggest that epigenetic mechanisms contribute to the transcriptional down-regulation of miR-221&222 in human H460 TRAIL sensitive cells. and a treatment of 5-aza-dC and a histone deacetylase inhibitor, trichostatinA, increased TRAIL resistance by up-regulation of miR 221&222.





### **Figure 11**

H460 cells were treated: **(A)** for 24 hrs at different concentrations of trichostatin A (TSA) (100, 200, 300, and 500 nM); **(B)** for 24 hrs with sodium butyrate (10, 25, 50, 75, and 100 mM); **(C)** daily for 72 hrs with 5'-Aza-2'-deoxycytidine (AZA) (1-5-10 mM); **(D)** for 24 hrs with 300nM of TSA or with 10mM 5'-AZA for 72hrs followed by 300nM TSA treatment for 24hrs. Cells were collected for RNA extraction. Northern blot analysis were performed as described in the methods, using miR-222 as probe. RNA loading control is also shown (EtBr = etidium bromide). **(E)** Cells were incubated for 24 or 48 hrs with 10mM of AZA. Northern blot analysis was performed as described in the methods. miR-221 was used as probe. An oligonucleotide complementary to the U6 RNA was used for normalization of the expression levels in the different samples. **(F)** H460 cells were treated for 24-48-72 hrs with 10  $\mu$ M AZA or with 300 nM TSA 24 hrs or with AZA for 72 hrs followed by TSA for 24 hr in the presence of TRAIL. 500.000 cells were then stained with FITC-conjugated annexin V antibody and propidium iodide (PI) and then subjected to flow cytometric analysis. miR221&222 overexpression, after AZA-TSA treatments, decreased TRAIL sensitivity in H460 cells.

### **p27<sup>Kip1</sup> and Kit expression in NSCLC**

We analyzed the predicted targets of the three most significantly overexpressed miRNAs (miR-222, miR-100, and miR-221) in TRAIL-resistant cells. The analysis was performed with three public available algorithms to predict human miR gene targets, i.e., MIRANDA, TARGETSCAN and PICTAR. In this study, we focused on miR-221 and -222, which are located close to each other on the short arm of the X chromosome. The concordant expression pattern in NSCLC suggested shared regulatory mechanisms for the expression of these two clustered miRs. They are up-regulated in TRAIL-resistant NSCLC cells and may promote TRAIL resistance by blocking expression of key functional proteins.

Different studies have demonstrated that both the pro-oncogene KIT and the tumor suppressor p27<sup>Kip1</sup> are miR-221&222 targets (25, 58-60). We thus investigated Kit and p27<sup>Kip1</sup> expression in NSCLC cells.

Western blotting of NSCLC cells with a monoclonal anti-Kit antibody revealed two bands of ~140kDa and 120kDa, corresponding to the mature fully glycosylated and the partially glycosylated Kit isoform, respectively (61) (Figure 12A). Interestingly, Kit protein was markedly up-regulated in sensitive, and down regulated in resistant, NSCLC cells.

FACS analysis, used for quantitative determination of cell surface Kit-receptor expression with specific antibodies against human Kit receptors (Figure 12B), confirmed the western blot results. Kit was a target of miR-221 and -222 in human NSCLC cells since its expression was up-regulated in CALU-1 cells upon anti-miR-222 transfection (Figure 12C). The p27<sup>Kip1</sup>

gene is a member of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors that function to negatively control cell cycle progression.

It binds to CDK2 and cyclin E complexes to prevent cell cycle progression from G1 to S phase. p27<sup>kip1</sup> also acts as a tumor suppressor and its expression is often disrupted in human cancers.

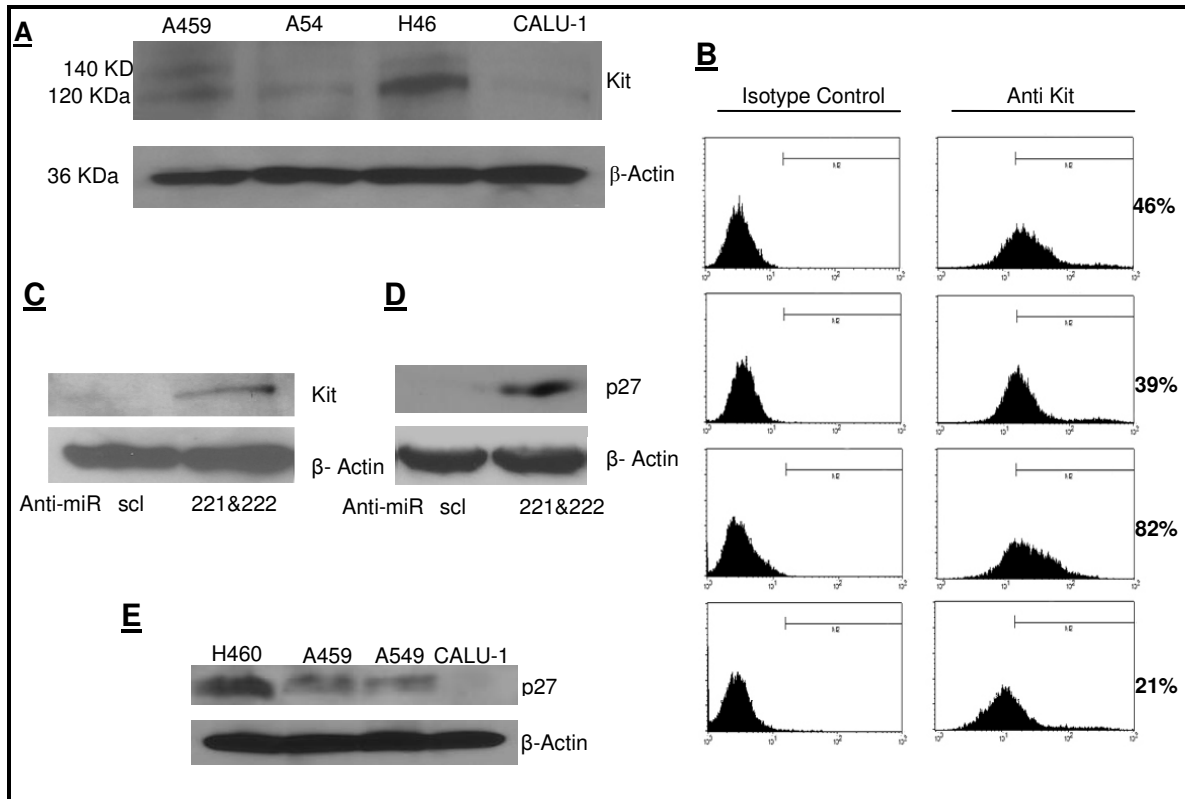
Decreased p27<sup>kip1</sup> levels have been correlated with tumor aggressiveness and poor patient survival (62, 63).

The low levels of p27<sup>kip1</sup> protein observed in many aggressive types of cancer are likely to be mediated by different mechanisms (62).

The abundance of p27<sup>kip1</sup> protein is largely controlled through a variety of post-transcriptional regulatory mechanisms (64, 65) among which are sequestration by cyclin D/CDK4 complexes, accelerated protein destruction and cytoplasmic retention (66) .

However, several studies have indicated that genes controlling the stability of p27<sup>kip1</sup> protein might not always account for its lower expression in cancer, and that p27<sup>kip1</sup> can also be regulated at the level of translation (67,68).

We next analyzed p27<sup>kip1</sup> expression in NSCLC cells. Interestingly, western blot analysis showed that p27<sup>kip1</sup> is clearly detectable in H460 cells, is reduced in A459 and A549 cells, and is very low or absent in CALU-1 cells (Figure 12E). Furthermore, as observed for Kit, the miR- 222 transfection resulted in an increase of p27<sup>kip1</sup> expression in CALU-1 cells (Figure 12D). A gene expression profiling in TRAIL-resistant and sensitive NSCLC cells, using oligonucleotide microarrays confirmed the down-regulation of Kit and p27<sup>kip1</sup> in CALU-1 resistant NSCLC cells (data not shown).



**Figure 12**

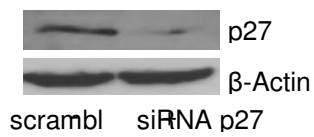
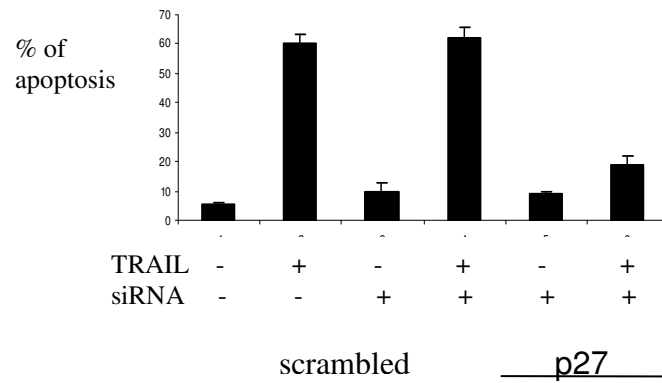
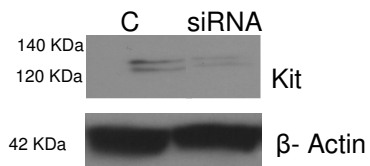
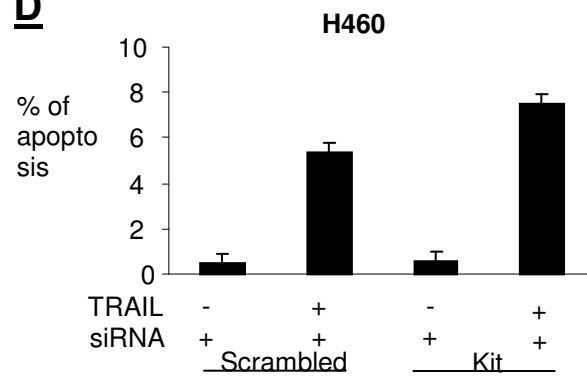
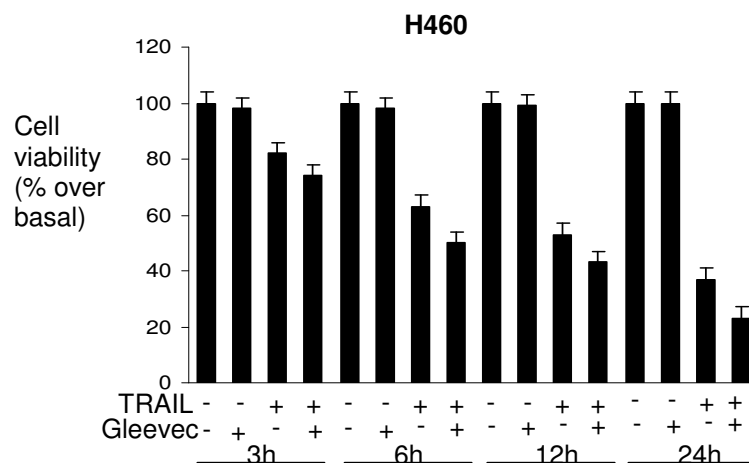
(A) Proteins from CALU-1, A459, A549 and H460 cells were extracted with RIPA buffer. Fifty  $\mu$ g of sample extract were resolved on 7.5% SDS-PAGE and transferred to Hybond-C extra nitrocellulose. Membranes were incubated with anti-Kit primary antibody (0.2  $\mu$ g/ml). Both surface and total Kit expression was higher in the H460 cells. (B) FACS analysis of cell surface Kit expression. Cells were stained with primary anti-human h-SCFR affinity purified goat IgG (1 $\mu$ g/ml) or mouse MAb 002 (Isotype control) followed by 10  $\mu$ l of secondary antibody, Fluorescein conjugated goat F(ab')<sub>2</sub>, as described in the methods section. % Gated: A459 (46%); A549(39%); H460(82%); Calu-1(21%). (C-D) CALU-1 cells were transfected with 100 nmol of anti miR-221&222 for 48 hours. Fifty micrograms of total extract was loaded onto 7.5-12% SDS-PAGE. The membrane was blotted with anti Kit (0.2  $\mu$ g/ml) and anti p27<sup>Kip1</sup> (1 $\mu$ g/ml) antibodies. Loading control was obtained with anti- $\beta$  actin antibody (1:5000). Anti miR-221&222 were able to increase Kit and p27<sup>Kip1</sup> expression. (E) p27<sup>Kip1</sup> expression in NSCLC- Fifty micrograms of total extract was loaded onto 12% SDS-PAGE. The membrane was

blotted with anti-p27<sup>Kip1</sup> (1µg/ml) antibody. Loading was controlled with an anti-β actin antibody (1:5000).

**Role of the miR221&222 targets, p27<sup>Kip1</sup> and Kit, on TRAIL-mediated apoptosis in NSCLC-** To investigate whether p27<sup>Kip1</sup> or Kit were involved in TRAIL resistance, we down-regulated these genes with specific siRNAs and then evaluated TRAIL sensitivity. Specific anti-Kit or anti-p27<sup>Kip1</sup> siRNAs induced a reduction of endogenous expression of Kit or p27<sup>Kip1</sup> proteins by about 70% (Figure 13A and C). In order to evaluate the role of p27<sup>Kip1</sup> in these cells, we measured survival after treatment with TRAIL. As shown in Figure 13B, anti-p27<sup>Kip1</sup> siRNA transfection increased H460 cell resistance to TRAIL. Surprisingly, the treatment of H460 with the specific Kit siRNA did not induce resistance to TRAIL treatments in H460 cells but an increase in apoptotic cell death mediated by TRAIL treatment (Figure 13D). Kit expression is under miR 221&222 control but it's down regulation is not responsible to TRAIL resistance in H460 cells.

Kit function was also investigated by interfering with its kinase activity by incubation of the cells with the drug Imatinib (Gleevec). H460 cells were incubated with 4µM Imatinib for 48h, and then treated with TRAIL (400ng/ml) for 3, 6, 12, 24 hrs. As a negative control, cells were treated with TRAIL or Imatinib alone (Figure 13E).

Imatinib-TRAIL co-treatment induced an increase of TRAIL sensitivity in H460 cells. The effect was evident after only 3hrs of co-incubation. Imatinib alone did not induce significant apoptosis in NSCLC.

**A****B****C****D****E**

### **Figure 13**

(A) H460 cells were cultured to 80% confluence and transiently transfected with anti-p27<sup>Kip1</sup> siRNA, a pool of 4 target specific 20-25 nt siRNA designed to knock down gene expression. Fifty micrograms of total extract was loaded onto 12% SDS-PAGE. Western blot was assessed as previously described. (B) Annexin V and propidium iodide (PI) staining of H460 cells after transfection with anti-p27 siRNA. Interfering with p27 expression increased TRAIL resistance in H460 cells. Mean  $\pm$  SD of four independent experiments in duplicate (C) H460 cells were transiently transfected in p60 plates using LIPOFECTAMINE 2000 with siKit RNA for 48 hours. Western blot of cells transfected using anti Kit antibody. Anti-Kit siRNA induced a marked down-regulation of Kit expression in H460 cells. (D) Propidium iodide (PI) staining of apoptotic cells- TRAIL was added for 24 hours and cell death was evaluated by PI staining and FACS analysis. Experiments were done twice in triplicate. Interfering with Kit expression increased TRAIL sensitivity in H460 cells (E) H460 cells were treated with 4 $\mu$ M imatinib for 48hrs, and then TRAIL (400ng/ml) was added for 3,6,12,24 hrs. As negative control cells were treated with TRAIL (400ng/ml) and Imatinib (4  $\mu$ M) alone. Cell viability assay was determined like previously described. A representative experiment of four independent experiments is presented.



## DISCUSSION

Apoptosis-based anti-cancer therapies are designed to achieve tumor eradication through the use of death-inducing molecules capable of activating the apoptotic program selectively in neoplastic cells. Due to its specific toxicity for malignant cells, recombinant forms of TRAIL are among the most promising apoptosis-based anti-tumor agents (69, 70). Therapy based on the use of agonistic TRAIL receptor antibodies are now in phase 2 clinical trial in different kinds of cancer, included NSCLC (71). However, in a number of patients, tumor cells evade death signals generated by drugs through the activation of effective anti-apoptotic mechanisms (72, 73). The aim of the present study was to identify specific signatures as potential therapeutic targets for the TRAIL-resistant phenotype in non small cell lung carcinoma (NSCLC).

For this purpose, we analyzed miRs expression profile in TRAIL-resistant (CALU-1) and semiresistant NSCLC cell lines (A459, A549), *versus* a TRAIL sensitive cell line (H460). We identified five miRs up-regulated in the resistant cell lines (miR-222,-100,-221,-125b and -15b) and between these we further analyzed four of them with the highest fold change (miR-222,-100,-221 and -125b). This pattern was specific for NSCLC cells since we did not find the same pattern in breast cancer cells analyzed with the same array (data not shown).

Forced overexpression of the miR -222, -100 and -221, but not of miR-125b, in the sensitive H460 cells increased resistance to TRAIL in these cells, thus indicating that repression of their target proteins is implicated in causing TRAIL resistance. Kept together our results show that the sensitivity of a cancer cell to a defined external signal is dictated by the expression of a small number of microRNAs.

It has recently described that miRNAs are under epigenetic control (74). The epigenetic silencing of these miRs modulate the activity of oncogenes (the cycline dependent kinase, CDK6) and tumor suppressor genes (retinoblastoma protein, Rb). In the present study we demonstrate that expression levels of at least two of these miRs, miR221&222, are maintained low in the TRAIL sensitive H460 cells by epigenetic modifications. Indeed, both demethylating agents and HDACis upregulated the levels of miR221&222 in a dose- and time-dependent manner. On the other hand, several studies demonstrate that HDACis treatment enhances TRAIL sensitivity in a number of different cancers gaining importance as potential anticancer drugs (47, 75). A plausible explanation for this apparent discrepancy likely relies on the pleiotropic effects of these agents on gene expression.

Further, in order to support the involvement of miR-mediated regulation of protein levels in TRAIL resistance, we investigated the potential protein targets of miRs identified in our screening. We focused on the two highly related miRs, miR-221 and -222, that recognize several predicted target genes involved in intracellular signaling (and cell death) and thus good candidate regulators of cell response to TRAIL.

Recent reports revealed that the receptor tyrosine kinase Kit and the cycline-dependent kinase inhibitor, p27<sup>kip1</sup>, are both miR-221 and -222 functional targets (25, 58-60). Here we demonstrate that silencing of p27<sup>kip1</sup>, but not of Kit, increases TRAIL resistance. This result well support the implication of miR-221 and -222 in determining the resistant/sensitive phenotype in NSCLC cells, and indicate p27<sup>kip1</sup> among target proteins that contribute to maintain cell sensitivity to TRAIL-induced cell death. Imatinib (Gleevec) is a tyrosine kinase inhibitor that targets Kit, plated-derived growth factor receptors (PDGFRs), and c-Abl (76). Hamai et al. (77), described that Imatinib treatment of melanoma cells was able to enhance TRAIL-induced cell death. In good agreement, we show that Gleevec further increases TRAIL-induced apoptosis in the H460 cells as well as in the CALU-1 cell line that do not express Kit, likely acting on other tyrosine kinase targets. Taken together these results indicate that even though miR-221 and -222 regulate the levels of both p27<sup>kip1</sup> and Kit proteins their effects on TRAIL sensitivity are mainly mediated by p27<sup>kip1</sup>. However, it seems plausible that silencing of other additional targets of miR-221 and -222 contribute to TRAIL resistance in NSCLC cells.

miR-221 and 222-mediated down regulation of p27<sup>kip1</sup> has been implicated in maintaining a more aggressive cancer phenotype, thus indicating p27<sup>kip1</sup> as bona fide tumor suppressor (26). p27<sup>kip1</sup> is a member of cyclin-dependent kinases (cdk) inhibitory proteins with putative tumor suppressor functions (78). More recently, p27<sup>kip1</sup> has been described to play different roles depending on the cell type context and on its cytosolic or nuclear cellular localization (79, 80). The functions of p27<sup>kip1</sup> in the apoptotic process remain

unclear. Adenovirus-mediated transient overexpression of p27<sup>kip1</sup> was demonstrated to induce apoptosis in transfected cells (81, 82). Other reports describe p27 as anti-apoptotic gene (83, 84). Therefore, this suggests that the survival effects of p27<sup>kip1</sup>, are cell-type specific and may be mediated by p27- effects on anti-apoptotic proteins expression (83, 85). Whether similar molecular mechanisms underlie the increase in TRAIL resistance upon silencing of p27<sup>kip1</sup> remain to be further investigated.

In conclusion, our results demonstrate for the first time that the intracellular levels of few miRs may modulate sensitivity of a cancer cell to a death receptor ligand with important implications in the design of new therapeutic agents.

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**Table 1**

has-miR-221	Xp11.3	5.9
has-miR-125b	11q23-24	5.4
has-miR-15b	3q26.1	3.6
hasmiR96	7q32.2	0.5
has-miR-9	1q23.1	0.072

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**Table-1-** All differentially expressed miRs have  $q < 0.01$  (false positive rate).

T test  $p < 0.05$ . These miRs were identified by PAM as predictor of NSCLC with the lowest misclassification error. All the miRs, except miR-9 and -96, are up-regulated in the TRAIL resistant cells compared to the TRAIL-sensitive one.

## Published articles:

Stassi G; Garofalo M; Zerilli M; Ricci-Vitiani L; Zanca C; Todaro M; Aragona F; Limite G; Petrella G; Condorelli G. (2005). **PED mediates AKT-dependent chemoresistance in human breast cancer cells.** Cancer Res 2005; 65: (15). August 1, 2005.

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Ciro Zanca, Michela Garofalo, Cristina Quintavalle, Giulia Romano, Mario Acunzo, Pia Ragno, Nunzia Montuori, Maria Rosaria Incoronato, Luigi Tornillo, Daniel Baumhoer, Luigi Terracciano, and Gerolama Condorelli **PED mediates TRAIL resistance in human non-small cell lung cancer. Accepted.**